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Knocking Down Type 2 but Not Type 1 Calsequestrin Reduces Calcium Sequestration and Release in C_2C_{12} Skeletal Muscle Myotubes*

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We examined the roles of type 1 and type 2 calsequestrins (CSQ1 and CSQ2) in stored Ca^{2+} release of C_2C_{12} skeletal muscle myotubes. Transduction of C_2C_{12} myoblasts with CSQ1 or CSQ2 small interfering RNAs effectively reduced the expression of targeted CSQ protein to near undetectable levels. As compared with control infected or CSQ1 knockdown myotubes, CSQ2 and CSQ1/CSQ2 knockdown myotubes had significantly reduced stored Ca^{2+} release evoked by activators of intracellular Ca^{2+} release channel/ryanodine receptor (10 mM caffeine, 200 μM 4-chloro-*m*-cresol, or 10 mM KCl). Thus, CSQ1 is not essential for effective stored Ca^{2+} release in C_2C_{12} myotubes despite our *in vitro* studies suggesting that CSQ1 may enhance ryanodine receptor channel activity. To determine the basis of the reduced stored Ca^{2+} release in CSQ2 knockdown myotubes, we performed immunoblot analyses and found a significant reduction in both sarco/endoplasmic reticulum Ca^{2+}-ATPase and skeletal muscle ryanodine receptor proteins in CSQ2 and CSQ1/CSQ2 knockdown myotubes. Moreover, these knockdown myotubes exhibited reduced Ca^{2+} uptake and reduced stored Ca^{2+} release by UTP (400 μM) that activates a different family of intracellular Ca^{2+} release channels (inositol 1,4,5-trisphosphate receptors). Taken together, our data suggest that knocking down CSQ2, but not CSQ1, leads to reduced Ca^{2+} storage and release in C_2C_{12} myotubes.

Calcium release from intracellular stores is a key step in a wide variety of cellular processes (1–3). In striated muscles, the release of stored Ca^{2+} from the sarcoplasmic reticulum (SR)[2] into cytosol by intracellular Ca^{2+} release channels known as ryanodine receptors (RyRs) initiates muscle contraction. Subsequently, the sequestration of cytosolic Ca^{2+} back into the SR by the sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA) leads to muscle relaxation (3). In addition to RyRs and SERCA, a number of SR proteins also play a significant role in Ca^{2+} storage and release and therefore may be important in striated muscle function. In this regard, the high-capacity, low-affinity Ca^{2+}-binding protein calsequestrin (CSQ) has drawn increased attention. Although CSQ is thought to increase Ca^{2+} storage capacity of SR in striated muscles (4–6), more recent studies have suggested that CSQ may also affect stored Ca^{2+} release by modulating RyR channel activities directly (7–9) or indirectly (10–12).

Murine muscles express two CSQ isoforms that share ~60% amino acid sequence homology (13). Crystal structure analysis has shown that the two CSQ isoforms form linear polymers as the Ca^{2+} concentration is increased (14). Type 1 CSQ (CSQ1) is expressed in fast-twitch skeletal muscle and is concentrated in the terminal cisternae of SR (15) where it interacts with triadin, an RyR-associated protein (8, 16). Consistent with its role in Ca^{2+} storage, overexpression of CSQ1 in skeletal muscle-derived C_2C_{12} myotubes increased Ca^{2+} store size (17). In addition, CSQ1 has also been shown to modulate skeletal muscle RyR (RyR1) channel activity directly (7–9) as well as through RyR-associated proteins such as triadin and junction (11, 18). Preliminary studies with a knock-out mouse model, null for CSQ1, have suggested that CSQ1 is an important determinant of skeletal muscle triad (SR-T-tubule) structure (19). Slow-twitch skeletal muscle co-express CSQ1 and type 2 CSQ (CSQ2) (20–22). In cardiac myocytes, CSQ2 forms a large multiprotein complex that includes the cardiac RyR (RyR2), triadin, and junctin (23). Overexpression of CSQ2 in cardiac myocytes increased SR Ca^{2+} levels as well as impaired SR Ca^{2+} release (24–26). A deficiency in CSQ2 (27) and a CSQ2 missense mutation (D307H) (28) both caused catecholamine-induced polymorphic ventricular tachycardia in humans. The importance of CSQ2 in skeletal muscle, however, is not well understood.

To better understand the roles of CSQ1 and CSQ2 in stored Ca^{2+} release of skeletal muscle, we used siRNAs to knockdown CSQ1 and/or CSQ2 in 8-day-old C_2C_{12} myotubes that otherwise robustly expressed both CSQ isoforms. After confirming the efficacies as well as specificities of our gene-silencing approach, RyR channel activities in the presence or absence of each CSQ isoform were examined using [^{3}H]ryanodine binding and single channel recordings. In addition, stored Ca^{2+} release in different experimental groups of C_2C_{12} myotubes evoked by activators of RyRs was assessed in the absence of extracellular Ca^{2+}. Although our *in vitro* studies suggest that CSQ1 may enhance RyR channel activity, CSQ1 knockdown myotubes unexpectedly had preserved stored Ca^{2+} release evoked by activators of RyRs. In contrast, CSQ2 and CSQ1/CSQ2 knockdown myotubes had significantly reduced stored Ca^{2+} release to the same RyR activators. Our subsequent data suggest that CSQ2, but not CSQ1, maintains type 1 SERCA (SERCA1) protein expression and Ca^{2+} sequestration activity in C_2C_{12} myotubes. Knocking down CSQ2 therefore leads to reduced Ca^{2+} storage and thus reduced stored Ca^{2+} release in these cells. In addition, a deficiency in CSQ2 may lead to reduced RyR1 expression in C_2C_{12} myotubes thus further compromising their stored Ca^{2+} release. A preliminary report of this work has been presented in abstract form (29).

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[2] The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; RyR1, type 1 RyR; RyR2, type 2 RyR; RyR3, type 3 RyR; CSQ1, type 1 calsequestrin; CSQ2, type 2 calsequestrin; SERCA1; type 1 sarco/endoplasmic reticulum Ca^{2+}-ATPase; siRNA, small interfering RNA; rAAV, recombinant adeno-associated virus; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; KRH, Krebs-Ringer-Henseleit; PIPES, 1,4-piperazinediethanesulfonic acid; DHPR, dihydropyridine receptor.

EXPERIMENTAL PROCEDURES

Materials—Immature C_2C_{12} skeletal muscle cells (myoblasts) derived from normal adult C_3H mouse leg muscles were purchased from ATCC (Manassas, VA). Double strand recombinant adenoassociated viral (rAAV) vector was generously provided by Dr. Douglas McCarty (University of North Carolina, Chapel Hill). Fluo 4-AM was purchased from Molecular Probes (Eugene, OR) and Fura 2-AM was purchased from TEF LABS (Austin, TX). Goat polyclonal anti-IP_3R2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal anti-IP_3R3 from BD Biosciences, and anti-junctin from ProSci Incorporated (San Diego, CA). Mouse monoclonal anti-RyR1/RyR3 IgG (34C clone) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). A rabbit polyclonal IgG raised against a 13-amino acid region specific for RyR3 and a C-terminal cysteine (KKRRRGQKVEKPEC) was prepared as described previously (30). All other primary antibodies were purchased from Affinity Bioreagents (Golden, CO). [^{3}H]Ryanodine was obtained from PerkinElmer Life Sciences. Phospholipids were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were obtained from Sigma unless specified otherwise.

Cell Culture—C_2C_{12} myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1× antibiotics/antimycotics. Myoblasts were seeded at a concentration of 6×10^5 cells per 10-cm plate and cultured for 48 h to reach 100% confluence (defined as day 0 myotubes). To induce myogenic differentiation, the growth medium was then changed to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1× antibiotics/antimycotics) on day 0. The differentiation medium was changed everyday subsequently.

Construction of Vector, Packaging, and Purification of rAAV—The oligonucleotides encoding the specific siRNA for each CSQ isoform were inserted into pSilencer-1.0 vector (Ambion, Austin TX) downstream of the U6 promoter using ApaI and EcoRI sites. U6 promoter-driven expression cassettes were inserted into rAAV vector (ptrs-U1a-RFP-U6) using the KpnI and NotI sites. The ptrs-U1a-RFP-U6 vector contains red fluorescent protein and was kindly provided by Dr. Douglas McCarty (University of North Carolina, Chapel Hill). The resulting vectors are termed rAAV-CSQ1 and rAAV-CSQ2. The sequences of oligonucleotides encoding the CSQ1 and CSQ2 siRNA were 5′-CTGAAGAAGACAGCGTTTATTCAAGAGATAAACGCTGTCTTCTTCAG-3′ and 5′-CAGTGGAGATCGTGAATAATTCAAGAGATTATTCACGATCTCCACTG-3′. BLAST searches confirmed that the selected oligonucleotide sequences were not homologous to any other genes. A control sequence of 5′-TTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAA-3′ was used to construct rAAV-control as a negative control. Serum type 2 double strand rAAVs were produced by the triple plasmid cotransfection method and purified by ammonium sulfate precipitation and on cesium chloride gradients (31, 32).

Recombinant AAV Transduction—For rAAV transduction, C_2C_{12} myoblasts were seeded at a concentration of 6×10^5 cells per 10-cm plate or 1×10^5 per well in 6-well plates. About 24 h after seeding, myoblasts were infected with rAAV carrying the CSQ-siRNA silencing cassette at 1×10^4 particles/cell and transferred into differentiation medium 24 h after infection. The differentiation medium was changed everyday subsequently.

Immunoblot and Immunofluorescence Analyses—Unless otherwise indicated, 8-day-old C_2C_{12} myotubes grown on 6-well plates were harvested, washed twice with cold phosphate-buffered saline, lysed in RIPA buffer plus protease inhibitors (Complete Mini, Roche Diagnostics), and centrifuged at $12{,}000 \times g$ for 10 min to remove insoluble material. Protein concentrations were determined using BCA assay. Twenty μg of lysate was separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies for CSQ1, CSQ2, triadin 95, junctin, α1-DHPR, RyRs, IP_3Rs, and SERCA1. Western blots were developed using 3,3′-diaminobenzidine or enhanced chemiluminescence and quantified using Kodak Digital Science ID Image Analysis Software. For immunofluorescence analysis, 8-day-old C_2C_{12} myotubes fixed with 3% paraformaldehyde were permeabilized with buffer containing 20 mM HEPES, pH 7.4, 0.3 M sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100, and incubated with anti-CSQ1 or anti-CSQ2 antibody, followed by incubation with Alexa Fluor 488-labeled goat anti-mouse or goat anti-rabbit antibody. Images were analyzed by confocal microscopy.

Preparation of Membrane Fractions—C_2C_{12} myotubes grown on 10-cm tissue culture dishes were washed twice with 3 ml of ice-cold phosphate-buffered saline containing 5 mM EDTA and harvested in phosphate-buffered saline containing 5 mM EDTA and protease inhibitors by removal from the plates by scraping. Cells were collected by centrifugation, resuspended in the above solution without EDTA, again pelleted, and stored at −80 °C. A crude membrane fraction was obtained by homogenizing the cells in 0.15 M KCl, 20 mM imidazole, pH 7, solution containing 0.3 M sucrose, 0.1 mM EDTA, 1 mM glutathione (oxidized), and protease inhibitors with the use of a Tekmar homogenizer (6 s at 13,500 rpm). Cell homogenates were centrifuged for 30 min at $100{,}000 \times g$, pellets were resuspended in 0.15 M KCl, 20 mM imidazole, pH 7, solution containing 0.3 M sucrose and protease inhibitors, and stored at −135 °C. Proteoliposomes containing purified rabbit skeletal muscle RyR1 ion channels were prepared in the presence of protease inhibitors (33).

Purification of Calsequestrins—Rabbit skeletal muscle (6) and canine cardiac muscle (34) CSQs were purified as described.

Stored Ca^{2+} Release—Stored Ca^{2+} release was determined using the fluorescent Ca^{2+} indicator dye Fluo 4-AM. 8-Day-old C_2C_{12} myotubes grown on glass coverslips were washed three times with phosphate-buffered saline and loaded with 5 μM Fluo 4-AM for 1 h at 37 °C in Krebs-Ringer-Henseleit (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 6 mM glucose, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, and 25 mM HEPES, pH 7.4). After loading, cells were rinsed with KRH buffer to remove non-hydrolyzed fluorophore and kept in KRH buffer for 30 min to complete de-esterification. Individual cells in Ca^{2+}-free KRH buffer (2 mM $CaCl_2$ was replaced by 0.5 mM EGTA) were defined as regions of interest, and average fluorescence was measured by using the program ImageMaster (Photon Technology International, Lawrenceville, NJ). Resting calcium levels were monitored with Fura-2. Cells were loaded with 5 μM Fura 2-AM and excited alternatively at 340 and 380 nm. The fluorescence emission was collected at 510 nm. Resting intracellular calcium concentrations were calculated using the equation: $[Ca^{2+}]_i = K_d(F_{380\,max}/F_{380\,min})(R - R_{min})/(R_{max} - R)$. A dissociation constant (K_d) of 224 nM (35, 36) was used for the binding of calcium to Fura-2 at 37 °C. R_{max} and R_{min} were determined in each experimental group by the consecutive addition of 10 μM ionomycin and 50 mM EGTA.

Restoring Ca^{2+} Release in CSQ2 Knockdown Myotubes—To determine whether CSQ2 is specifically responsible for the decreased stored Ca^{2+} release of C_2C_{12} myotubes treated with CSQ2 siRNA, we performed additional experiments. We first amplified CSQ2 cDNAs from a mouse skeletal muscle cDNA library (Clontech) by PCR and then constructed the CSQ2 cDNAs into PcIneo vector using NheI and NotI sites. 3 μg of PcIneo-CSQ2 plasmid DNA, 1 μg of ptrs-U1a-RFP vector (as a reporter), and 10 nM chemically synthesized siRNA specific for CSQ2 (see above targeting sequence) (Qiagen, Valencia, CA) were then mixed in 10 μl of Lipofectamine 2000 (Invitrogen) and co-transtected into C_2C_{12} myoblasts in each well of the 6-well plate. In two other groups of

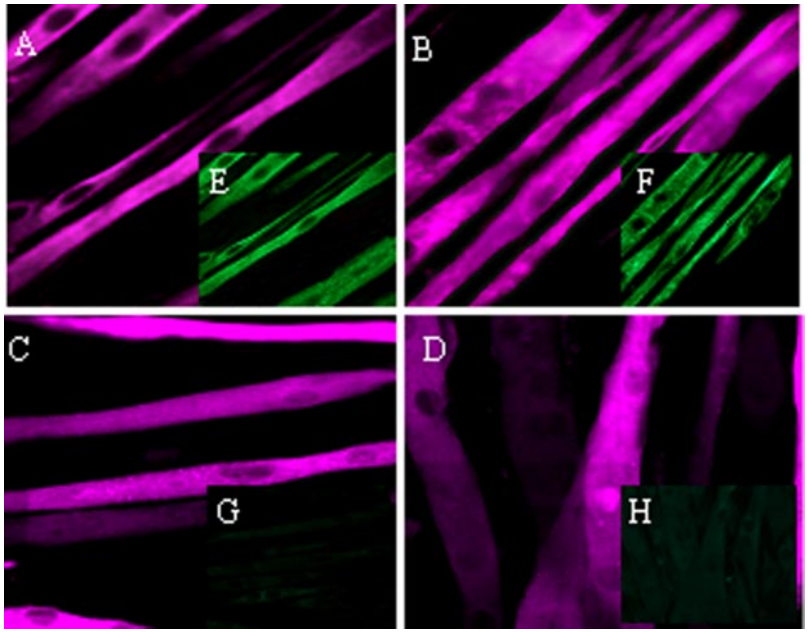

FIGURE 1. **Knocking down CSQ1 and CSQ2 in 8-day-old C_2C_{12} myotubes.** The high efficacies of rAAV transduction are indicated by the expression of double strand RFP in C_2C_{12} myotubes infected with rAAV-control (*A* and *B*) or rAAV-CSQ1 plus rAAV-CSQ2 (*C* and *D*). *Insets* show the localization of CSQ1 (*E*) and CSQ2 (*F*) in rAAA-control infected myotubes and the absence of CSQ1 (*G*) and CSQ2 (*H*) in rAAV-CSQ1/rAAV-CSQ2-infected C_2C_{12} myotubes.

C_2C_{12} myoblasts, PcIneo-CSQ2 was replaced by the same amount of PcIneo vector in one group and CSQ2 siRNA was omitted in the other group. The transfection efficiency was about 5%. C_2C_{12} myoblasts were then allowed to differentiate over 8 days and myotubes with red fluorescence were examined for caffeine-induced stored Ca^{2+} release as described above.

[^{3}H]Ryanodine Binding—Unless otherwise indicated, crude membrane fractions prepared from C_2C_{12} cells were incubated with 2.5 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 250 mM KCl, 150 mM sucrose, 1 mM glutathione (oxidized), 20 μM leupeptin, and 200 μM Pefabloc, and the indicated free Ca^{2+} concentrations. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 20 h at 24 °C, aliquots of the samples were diluted with 8.5 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with 3× 5 ml of ice-cold 0.1 M KCl, 1 mM K-PIPES, pH 7.0. Radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

B_{max} values of [^{3}H]ryanodine binding were determined by incubating membranes for 4 h at 24 °C with a saturating concentration of [^{3}H]ryanodine (30 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 20 μM leupeptin, 200 μM Pefabloc, and 100 μM Ca^{2+}. Specific [^{3}H]ryanodine binding was determined as described above.

$^{45}Ca^{2+}$ Uptake—ATP-dependent $^{45}Ca^{2+}$ uptake by C_2C_{12} membranes was determined using a filtration method. $^{45}Ca^{2+}$ uptake was initiated by placing membranes in 0.15 M KCl, 20 mM imidazole, pH 7.0, solution containing 5 mM ATP, 8 mM Mg^{2+}, 5 mM Koxalate (a Ca^{2+} precipitating agent to increase Ca^{2+} uptake capacity (37)), 10 μM ruthenium red (to inhibit RyRs (38)), 5 mM NaN_3 (to inhibit mitochondrial Ca^{2+} uptake), 1 mM EGTA, and $^{45}Ca^{2+}$ to yield a free Ca^{2+} concentration of 0.5 μM. To obtain $^{45}Ca^{2+}$ uptake rates, aliquots were placed at 2.5, 5, and 10 min on 0.45 μm Millipore filters under vacuum and rinsed with three 3-ml volumes of ice-cold 0.175 M KCl, 5 mM imidazole, pH 7.0, solution. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting.

Single Channel Recordings—Single channel measurements were performed using Mueller-Rudin-type planar lipid bilayers containing a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (25 mg of total phospholipid/ml of *n*-decane) (39). SR vesicles of 8-day-old C_2C_{12} myotubes were added to the *cis* (SR cytosolic side) chamber of a bilayer apparatus and fused in the presence of an osmotic gradient (250 mM *cis* KCl, 20 mM *trans* KCl in 20 mM K-HEPES, pH 7.4, with 2 μM Ca^{2+}). Amounts of membranes were adjusted to obtain single channel activities not more than 30 min after their addition. After appearance of channel activity, *trans* (SR lumenal) KCl concentration was increased to 250 mM to prevent further fusion of membranes. The *trans* side of the bilayer was defined as ground. The large cytosolic regulatory region of the channels faced the *cis* chamber in a majority (>98%) of the recordings (40). Purified canine cardiac and rabbit skeletal muscle CSQs were added to the *trans* chamber. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed as described (40).

Biochemical Assays and Data Analyses—Free Ca^{2+} concentrations were obtained by including in the solution the appropriate amounts of Ca^{2+} and EGTA as determined using the stability constants and published computer program (41). Free Ca^{2+} concentrations of ≥1 μM were verified with the use of a Ca^{2+} selective electrode.

Results are given as mean ± S.E. Significance of differences in data ($p < 0.05$) were determined using Student's *t* test.

RESULTS

Efficiencies and Specificities of Knocking Down CSQ1 and CSQ2 in C_2C_{12} Myotubes—C_2C_{12} myoblasts were infected with rAVV vectors containing the sequences for red fluorescent protein, U6 promoter (ptrs-U1a-RFP-U6), and CSQ1 or CSQ2 specific siRNA. These C_2C_{12} myoblasts were then allowed to differentiate into multinucleated myotubes over 8 days (Fig. 1) as described under "Experimental Procedures." In addition, C_2C_{12} myoblasts were doubly infected to knockdown both CSQ isoforms or were infected with rAAV vector that contained a control oligonucleotide sequence. Transduction efficiencies among these four groups of C_2C_{12} myotubes were all near 100% as judged by the red fluorescent protein expression of the myotubes. As shown Fig. 1, *A–D*, every myotube expressed the red fluorescent protein when examined using confocal microscopy.

In our preliminary studies, we had found that C_2C_{12} myotubes robustly expressed both CSQ isoforms after 6 or more days of culture in

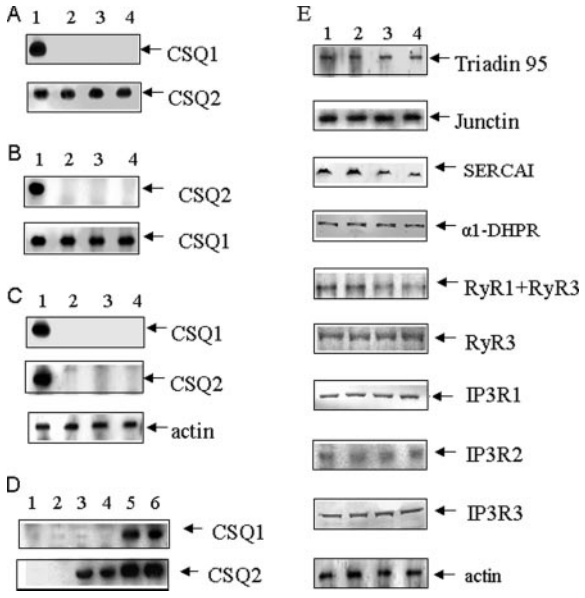

FIGURE 2. **Immunoblot analyses of C_2C_{12} myotubes.** CSQ1 and CSQ2 levels in C_2C_{12} myotubes infected with rAAV-CSQ1 (*A*), rAAV-CSQ2 (*B*), or rAAV-CSQ1/rAAV-CSQ2 (*C*) were examined using immunoblot techniques. *Lane 1*, C_2C_{12} myotubes infected with rAAV-control. *Lanes 2–4*, three independent groups of 8-day-old myotubes infected with rAAV-CSQ. *D*, time course of CSQ expression in control, rAAV-CSQ1-, or rAAV-CSQ2-infected C_2C_{12} myotubes. *Lanes 1*, *3*, and *5* are control C_2C_{12} myotubes at days 1, 3, and 6. *Lanes 2*, *4*, and *6* are days 1, 3, and 6 C_2C_{12} myotubes infected by rAAV-CSQ2 (*upper row*) and rAAV-CSQ1 (*lower row*). *E*, immunoblots were performed on 8-day-old C_2C_{12} myotube lysate. *Lane 1*, C_2C_{12} myotubes infected with rAAV-control. *Lanes 2–4*, C_2C_{12} myotubes infected with rAAV-CSQ1, rAAV-CSQ2, and rAAV-CSQ1/rAAV-CSQ2, respectively.

differentiation medium (*e.g.* Fig. 2*D*). The cellular distribution of CSQ isoforms in 8-day-old myotubes was then examined after labeling these cells with anti-CSQ1 or anti-CSQ2 antibody and followed by Alexa 488-conjugated secondary bodies. As shown in Fig. 1, *E* and *F*, the two CSQ isoforms had a similar cellular distribution in 8-day-old (control) myotubes. Transductions with rAAV-CSQ1 and rAAV-CSQ2 reduced the corresponding CSQ isoform to nearly undetectable levels in 8-day-old C_2C_{12} myotubes. Representative results are shown in Fig. 1, *G* and *H* (background green fluorescence is barely noticeable).

The efficacies of knocking down the two CSQ isoforms in C_2C_{12} myotubes were confirmed by immunoblot analyses. As with our immunofluorescence studies, CSQ1 and CSQ2 proteins (both had an apparent molecular mass of ~60 kDa on gels) in myotubes were reduced to undetectable levels by their corresponding siRNAs (Fig. 2, *A–C*). Also notable were the specificities of CSQ siRNAs. That is, the level of CSQ2 protein was not affected in C_2C_{12} myotubes infected with rAAV-CSQ1 and similarly the level of CSQ1 protein was not affected in C_2C_{12} myotubes infected with rAAV-CSQ2 (Fig. 2, *A* and *B*). These results clearly show that infecting C_2C_{12} myoblasts with rAAV encoding CSQ1- or CSQ2-specific siRNA sequence results in the specific knockdown of corresponding CSQ protein in C_2C_{12} myotubes. These results also validated the isoform specificities of anti-CSQ antibodies used in our immunoblot and immunofluorescence studies. Furthermore, these results indicate that knocking down one particular CSQ isoform does not lead to a compensatory overexpression of the other isoform.

The expression levels of CSQ1 and CSQ2 proteins in different stages of C_2C_{12} myotubes were investigated using a combination of immunoblot and protein densitometry analyses. Immunoblot studies revealed that CSQ1 and CSQ2 were absent in 1-day-old myotubes (Fig. 2*D*, *lane 1*). CSQ2 was first detected in 3-day-old myotubes (*lane 3*) but by day 6 both CSQ isoforms were easily detectable (*lane 5*). The expression level of CSQ1 was not altered in 3- or 6-day-old C_2C_{12} cells infected with rAAV-CSQ2 (Fig. 2*D*, *upper panel*, compare *lanes 3* and *4*, and *5* and *6*). Conversely, transduction with rAAV-CSQ1 had no noticeable effect on CSQ2 levels in 3- and 6-day-old C_2C_{12} myotubes (Fig. 2*D*, *lower panel*).

We were able to estimate the relative expression of CSQ isoforms in 8-day-old myotubes because our knockdown method is highly efficacious and knocking down one CSQ isoform does not lead to a compensatory overexpression of the other isoform (Fig. 2, *A–D*). Densitometry analyses of the Coomassie Blue-stained gels showed that, as compared with rAAV-control infection, rAAV-CSQ1 infection reduced the CSQ protein band by 61 ± 4% in 8-day-old myotubes (not shown). In comparison, rAAV-CSQ2 transduction reduced the CSQ protein band by 31 ± 5% ($n = 3$). These data suggest that 8-day-old C_2C_{12} myotubes expressed CSQ1 and CSQ2 at a protein ratio of ~2:1 despite the apparent higher signals of CSQ2 bands in immunoblots (Fig. 2*D*).

Regulation of RyR by CSQ—To investigate how the two CSQ isoforms may regulate channel activities of RyR1 and RyR3 (8-day-old C_2C_{12} myotubes expressed both isoforms, Ref. 42, see also below) thereby affecting stored Ca^{2+} release in skeletal myotubes, we performed single RyR channel measurements with membranes isolated from 8-day-old C_2C_{12} myotubes doubly infected with rAAV-CSQ1 and rAAV-CSQ2 and therefore devoid of both CSQ1 and CSQ2 (Fig. 2*C*). The regulatory effects of each CSQ isoform as reflected by the changes in RyR channel open probabilities (P_o) were then determined by adding exogenous purified rabbit skeletal muscle CSQ1 to the *trans* (SR lumenal) side of lipid bilayer setup. Because the purified rabbit CSQ1 preparation also contained CSQ2 as a minor component as determined by immunoblot analysis, we also performed a limited number of experiments with purified canine calsequestrin that contained only CSQ2. Similarly, we studied membranes isolated from rAVV control-infected myotubes as comparison. To establish well defined cytoplasmic Ca^{2+} concentrations, K^+ rather than Ca^{2+} was used as the current carrier (43). Fig. 3*A* shows a representative single channel recording study that investigated the effects of 4 μM purified rabbit skeletal muscle CSQ on RyR P_o. Baseline P_o was recorded with 2 μM free Ca^{2+} and 1 mM ATP in the *cis* (cytosolic) side of bilayer chamber. Adding 1 mM Ca^{2+} to the *trans* (SR lumenal) chamber increased P_o recorded from both CSQ1/CSQ2 depleted and control membranes. The subsequent addition of 4 μM CSQ to the *trans* chamber, however, increased P_o from CSQ-knockdown but not from control membranes. Table 1 summarizes the averaged P_o values of RyRs isolated from control and CSQ1/CSQ2 knockdown myotubes without and with 1 mM lumenal Ca^{2+} and after the addition of 2–4 μM CSQ. The effect of CSQ was dependent on the lumenal concentration of Ca^{2+}. Adding CSQ in the presence of a low *trans* Ca^{2+} concentration (2 μM Ca^{2+}) had no effect on P_o from CSQ knockdown membranes but P_o was significantly enhanced by the subsequent addition of 1 mM Ca^{2+} (Fig. 3*B*, Table 1).

CSQ was less effective in enhancing channel activities when only CSQ1 isoform was knocked down. In the presence of 1 mM lumenal Ca^{2+}, addition of 2 μM CSQ1 increased CSQ1-depleted RyR channel activities 1.09 ± 0.03-fold ($n = 9$, $p < 0.05$) as compared with 1.75 ± 0.18-fold increase of P_o values of RyRs isolated from myotubes devoid of both CSQ1 and CSQ2 isoforms. The modest effect of CSQ1 on CSQ1-depleted channels suggests that CSQ2 likely remained with these channels and impeded access of CSQ1.

The regulatory effect of CSQ on RyRs appears to be dependent on other SR proteins such as triadin and junctin (18). Our single channel recording studies of purified rabbit skeletal muscle RyR1 indicated that 4 μM rabbit CSQ had no noticeable enhancing effect on RyR1 P_o even in the presence of 1 mM lumenal Ca^{2+} (data not shown).

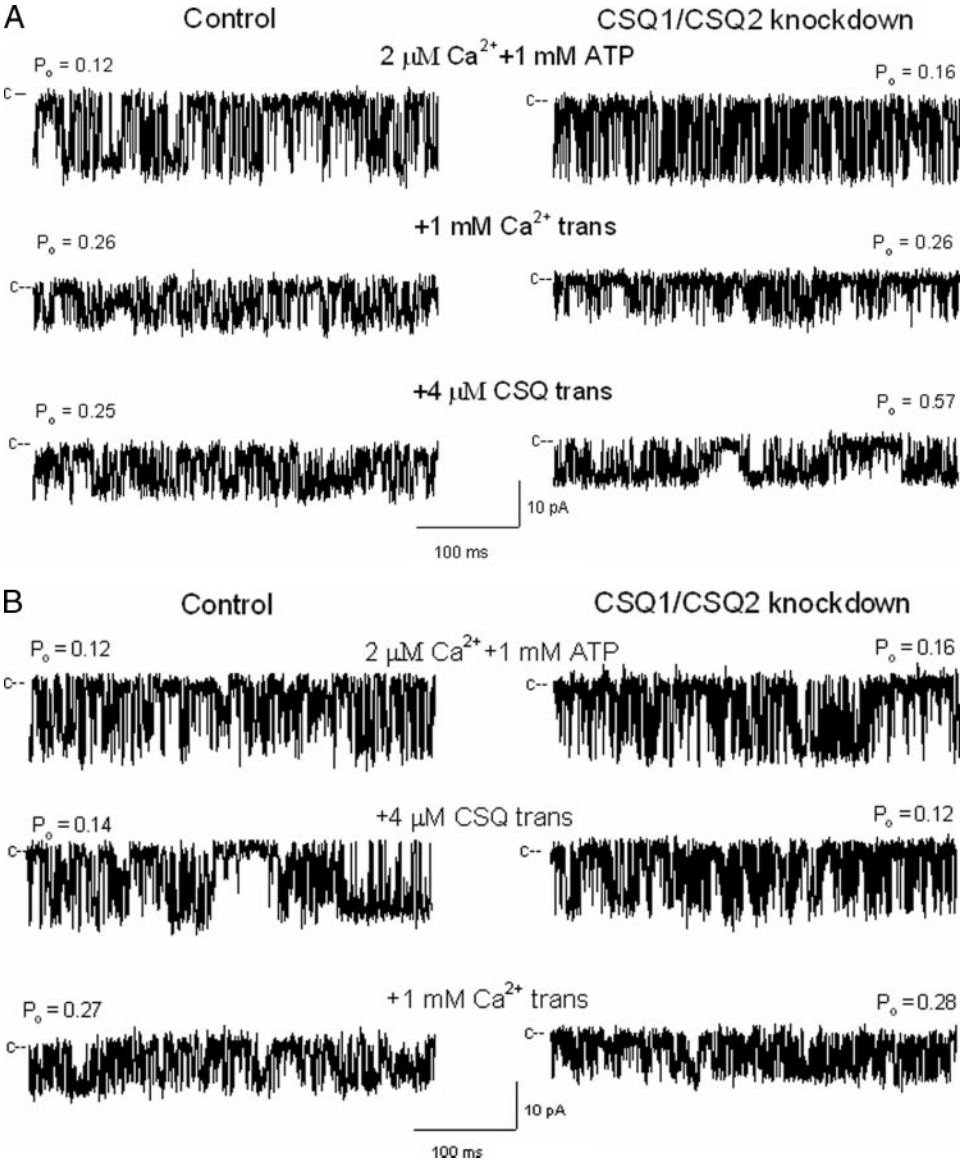

FIGURE 3. **Effects of rabbit skeletal CSQ on single RyR channel activity.** *A*, membranes isolated from C_2C_{12} myotubes infected with rAAV-control (*left*) and rAAV-CSQ1/rAAV-CSQ2 (*right*) were fused with lipid bilayer. Single channel currents (downward deflections from closed levels, *c*–) were recorded at −20 mV as described under "Experimental Procedures" in the absence (*top traces*) and presence of 1 mM Ca^{2+} *trans* (*middle traces*) and 1 mM Ca^{2+} *trans* plus purified rabbit skeletal muscle 4 μM CSQ *trans* (*bottom traces*). *B*, single channel currents were recorded as in *A* in the absence (*top traces*) and presence of 4 μM CSQ *trans* (*middle traces*) and 4 μM CSQ *trans* plus 1 mM Ca^{2+} *trans* (*bottom traces*).

TABLE 1

Effects of SR lumenal Ca^{2+} and purified rabbit skeletal muscle CSQ on RyR single channel activities from control and CSQ1/CSQ2 knockdown myotubes

SR lumenal rabbit skeletal muscle CSQ concentration was 2–4 μM. Data are the mean ± S.E. of number of experiments indicated in parentheses. Included in these are multiple channel recordings (3 of 9 and 2 of 5 in control; 3 of 9 and 3 of 4 in CSQ1/CSQ2 knockdown).

Additions	Control	CSQ1/CSQ2 knockdown
	P_o	
2 μM Ca^{2+} *cis* + 1 mM ATP *cis*	0.12 ± 0.03(9)	0.17 ± 0.02(9)
+1 mM Ca^{2+} *trans*	0.25 ± 0.06[a]	0.24 ± 0.04[a]
+1 mM Ca^{2+} *trans* + CSQ *trans*	0.25 ± 0.07[a]	0.40 ± 0.07[a,b]
2 μM Ca^{2+} *cis* + 1 mM ATP *cis*	0.16 ± 0.05(5)	0.21 ± 0.03(4)
+CSQ *trans*	0.14 ± 0.02	0.20 ± 0.04
+CSQ *trans* + 1 mM Ca^{2+} *trans*	0.26 ± 0.04[a,c]	0.33 ± 0.03[a,c]

[a] $p < 0.05$ *versus* 2 μM Ca^{2+} *cis* + 1 mM ATP *cis*.
[b] $p < 0.05$ *versus* 1 mM *trans* Ca^{2+}.
[c] $p < 0.05$ *versus* 2–4 μM CSQ *trans*.

In single channel recording studies, CSQ2 purified from canine cardiac muscle had a more modest enhancing effect on RyR channel activities as compared with purified rabbit skeletal muscle CSQ. In the presence of 1 mM lumenal Ca^{2+}, adding 2 μM CSQ2 caused a small (but significant) increase in CSQ1/CSQ2-depleted RyR single channel activities ($P_o = 0.24 \pm 0.05$ *versus* 0.29 ± 0.06, $n = 7$, $p < 0.05$).

The effects of rabbit CSQ on RyR channel activities were examined further. Fig. 4 shows that a fairly wide range of CSQ concentrations (0.4–4 μM) was effective in increasing channel open probability by 1.5–2-fold. A higher CSQ concentration of 12 μM returned P_o to near control levels. Control measurements showed that this decrease was not because of a change in lumenal Ca^{2+} concentration. Kinetic analysis showed that CSQ (in the presence of 1 mM lumenal Ca^{2+}) increased P_o by raising the number of single channel events without significantly changing the mean open times (Table 2). Taken together, the results of our single channel measurements suggest that rabbit CSQ (which contains CSQ1 as the major component), and to a smaller extent canine CSQ2, enhance C_2C_{12} myotube RyR channel activities in the presence of 1 mM luminal Ca^{2+} by increasing the transition rates from the closed to open states.

We also tested the possibility that CSQ knockdown affected the Ca^{2+} dependence of RyR activities isolated from CSQ knockdown and control myotubes, using the RyR-specific probe ryanodine (3, 44). Fig. 5 shows that knockdown of CSQ1, CSQ2, and both isoforms did not significantly alter the bimodal Ca^{2+} dependence of RyR activity.

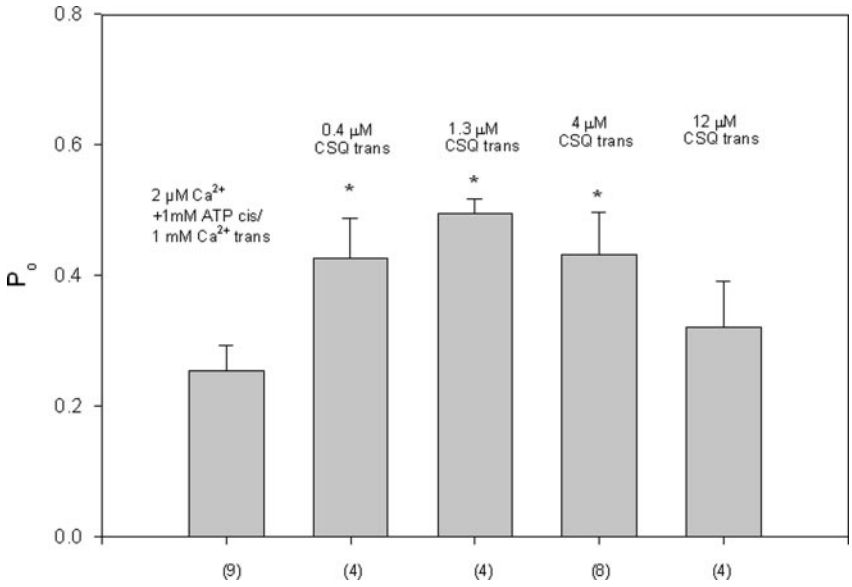

FIGURE 4. **Channel activities of RyR as a function of CSQ concentration.** Single channel activities were determined as described in the legend to Fig. 3 in the presence of the indicated concentrations of purified rabbit skeletal muscle CSQ and 1 mM *trans* (SR lumenal) Ca^{2+}. Data are the mean ± S.E. of four to nine experiments. *, $p < 0.05$ compared with control (without CSQ).

TABLE 2

Effects of SR lumenal Ca^{2+} and CSQ on kinetic parameters of single channel recordings

SR lumenal rabbit skeletal muscle calsequestrin (CSQ) concentration was 2–4 μM. Average normalized parameters (in the absence of 1 mM Ca^{2+} + CSQ) at 2 μM Ca^{2+} *cis* + 1 mM ATP *cis* were for control and CSQ1/CSQ2-depleted RyRs, respectively: $P_o = 0.13 \pm 0.05$ and 0.15 ± 0.04, Events/min = 7,763 ± 858 and 10,114 ± 2,284, $T_o = 1.00 \pm 0.33$ and 0.94 ± 0.14 ms, $T_c = 7.44 \pm 1.17$ and 6.67 ± 1.26 ms. Channel parameters were calculated from 6 recordings each that contained a single channel activity.

Additions	P_o	Events	T_o	T_c
		min^{-1}		*ms*
Control				
2 μM Ca^{2+} *cis* + 1 mM ATP *cis*	100	100	100	100
+1 mM Ca^{2+} *trans*	243 ± 38[a]	221 ± 34[a]	92 ± 9	34 ± 7[a]
+1 mM Ca^{2+} *trans* + CSQ *trans*	256 ± 34[a]	262 ± 40[a]	90 ± 7	35 ± 9[a]
CSQ1/CSQ2 knockdown				
2 μM Ca^{2+} *cis* + 1 mM ATP *cis*	100	100	100	100
+1 mM Ca^{2+} *trans*	154 ± 19[a]	220 ± 41[a]	82 ± 17	42 ± 6[a]
+1 mM Ca^{2+} *trans* + CSQ *trans*	277 ± 33[a,b]	296 ± 49[a]	116 ± 33	25 ± 6[a,b]

[a] $p < 0.05$ compared with 2 μM Ca^{2+} *cis* + 1 mM ATP cis.
[b] $p < 0.05$ compared with 1 mM Ca^{2+} *trans* addition.

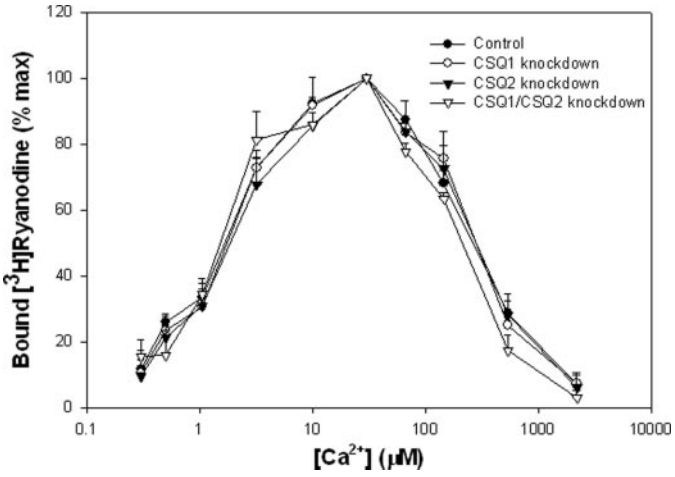

FIGURE 5. **Ca^{2+} dependence of [^{3}H]ryanodine binding to control and CSQ-knockdown C_2C_{12} myotubes.** Specific [^{3}H]ryanodine binding was determined in 250 mM KCl, 20 mM imidazole, pH 7.0, media containing 2.5 nM [^{3}H]ryanodine and the indicated concentrations of free Ca^{2+}. Data are mean ± S.E. of three to four experiments.

Stored Ca^{2+} Release in CSQ Knockdown Myotubes Provoked by Activators of Intracellular Ca^{2+} Release Channels—Because our single channel recording studies suggest that both CSQ1 and CSQ2 may enhance stored Ca^{2+} release by RyRs in C_2C_{12} myotubes, we then tried to correlate these results with the effects of CSQ knockdown on stored Ca^{2+} release in intact myotubes using the fluorescent Ca^{2+} indicator Fluo-4 in the absence of extracellular Ca^{2+}. In initial experiments using the fluorescent Ca^{2+} indicator Fura-2, we determined that there was no significant difference in the resting cytosolic Ca^{2+} concentration of control and each of the three CSQ-knockdown myotube groups (data not shown). Depolarization-induced, RyR-mediated stored Ca^{2+} release in 8-day-old myotubes was provoked by 10 mM KCl. Stored Ca^{2+} release, as reflected by the Fluo-4 fluorescence over baseline fluorescence ratio (F/F_o), was significantly smaller in CSQ2 and CSQ1/CSQ2 knockdown myotubes (Fig. 6*A*) as compared with the control myotubes. The peak F/F_o ratio was 3.2 ± 0.1 for the control group, 2.4 ± 0.2 for the CSQ2 knockdown group, and 2.2 ± 0.2 for the CSQ1/CSQ2 knockdown group (Table 3). In contrast, the peak F/F_o in CSQ1 knockdown myotubes induced by 10 mM KCl was comparable with the control myotubes. To confirm that RyR-mediated stored Ca^{2+} release is reduced in CSQ2 knockdown myotubes, we also treated the four groups of C_2C_{12} myotubes with caffeine (which activates RyR1 and RyR3 independent of the DHPR) or 4-chloro-*m*-cresol (which activates RyR1 but not RyR3 (45)) (the 8-day-old C_2C_{12} myotubes expressed both RyR1 and RyR3 (Fig. 2*E*)). Indeed, the Ca^{2+} responses of 8-day-old C_2C_{12} myotubes to 10 mM caffeine or 200 μM 4-chloro-*m*-cresol in the absence of extracellular Ca^{2+} were also significantly decreased in CSQ2 and CSQ1/CSQ2 but not in CSQ1 knockdown myotubes (Fig. 6, *B* and *C*, Table 3).

To determine whether the lack of CSQ2 is specifically responsible for the reduced stored Ca^{2+} release of C_2C_{12} myotubes treated with CSQ2 siRNA,

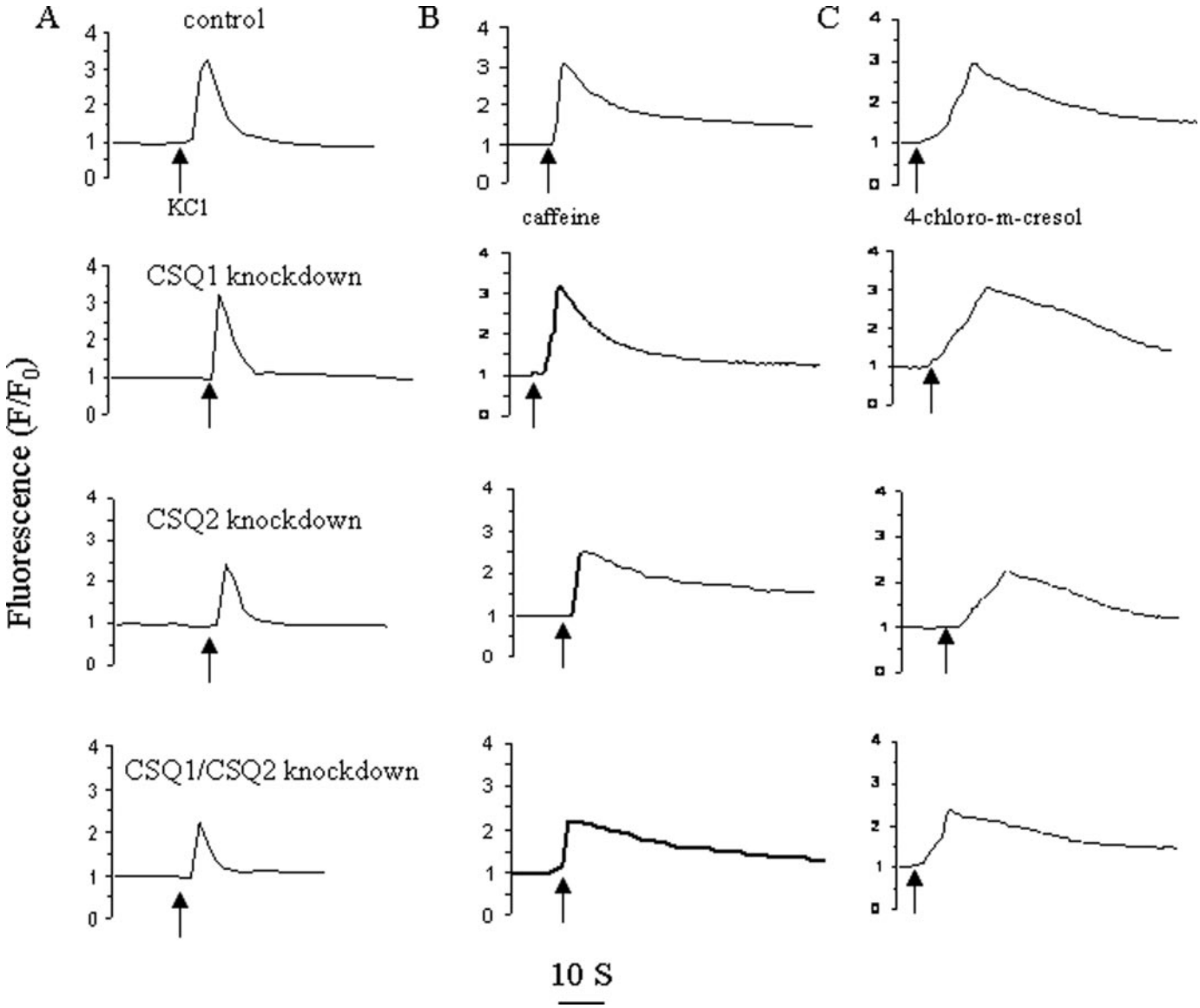

FIGURE 6. **Effects of CSQ knockdown on stored Ca^{2+} release in C_2C_{12} myotubes.** Depolarization (10 mM KCl addition) (*A*), caffeine (10 mM) (*B*), and 4-chloro-*m*-cresol (200 μM) (*C*) induced Ca^{2+} release were determined in Ca^{2+}-free KRH bath solutions as changes of Fluo 4 fluorescence (F/F_o) in C_2C_{12} myotubes infected with rAAV-control (*top traces*), rAAV-CSQ1 (*second traces*), rAAV-CSQ2 (*third traces*), and rAAV-CSQ1/rAAV-CSQ2 (*bottom traces*).

TABLE 3

Effects of CSQ knockdown on stored Ca^{2+} release in C_2C_{12} myotubes

The peak values of fluorescence increases (F/F_o) in C_2C_{12} myotubes were determined in Fluo 4-loaded cells in Ca^{2+}-free KRH buffer. Data are the mean ± S.E. of 8–16 experiments.

Addition	Control	Peak fluorescence (F/F_o)		
		CSQ1 knockdown	CSQ2 knockdown	CSQ1/CSQ2 knockdown
10 mM KCl	3.2 ± 0.1	3.1 ± 0.2	2.4 ± 0.2[a]	2.2 ± 0.2[a]
10 mM Caffeine	3.1 ± 0.1	2.9 ± 0.2	2.5 ± 0.3[a]	2.1 ± 0.2[a]
200 μM 4-Chloro-*m*-cresol	2.9 ± 0.1	2.9 ± 0.2	2.3 ± 0.1[a]	2.2 ± 0.2[a]
1 μM Thapsigargin	3.0 ± 0.1	3.0 ± 0.2	2.3 ± 0.1[a]	2.0 ± 0.1[a]
400 μM UTP	2.9 ± 0.2	2.9 ± 0.3	2.2 ± 0.2[a]	2.3 ± 0.1[a]

[a] $p < 0.05$ compared to control.

C_2C_{12} myoblasts were treated with the same CSQ2 siRNA but synthesized chemically with or without co-transfection with a plasmid containing the sequence of CSQ2. Another group of myotubes was used as control (see "Experimental Procedures"). In these experiments, the peak fluorescences (F/F_o) were 3.0 ± 0.1 ($n = 7$) for control myotubes and 2.1 ± 0.1 ($n = 8$) for myotubes treated with CSQ2 siRNA ($p < 0.05$). We found that the marked reduction in caffeine-induced stored Ca^{2+} release caused by CSQ2 siRNA is partially restored in myotubes co-transfected with CSQ2 siRNA and a plasmid containing the sequence of CSQ2 ($F/F_o = 2.7 \pm 0.1, n = 6, p < 0.05$ as compared with CSQ2 siRNA myotubes). These results suggest that the reduction in stored Ca^{2+} release in CSQ2 siRNA-treated myotubes was specific with regard to CSQ2 knockdown.

TABLE 4

B_{max} values of [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ uptake rates of membranes isolated from control and CSQ knockdown C_2C_{12} myotubes

The total number of [^{3}H]ryanodine binding sites (B_{max}) and $^{45}Ca^{2+}$ uptake rates of crude membrane fractions were determined as described under "Experimental Procedures." Data are the mean ± S.E. of three to eight experiments.

Sample	B_{max} of [^{3}H]ryanodine binding	$^{45}Ca^{2+}$ uptake rate
	pmol/mg protein	*nmol/mg protein/min*
Control	0.35 ± 0.04	18.6 ± 3.5
CSQ1 knockdown	0.35 ± 0.04	16.5 ± 3.4
CSQ2 knockdown	0.21 ± 0.03[a]	7.8 ± 2.3[a]
CSQ1/CSQ2 knockdown	0.24 ± 0.02[a]	8.4 ± 2.1[a]

[a] $p < 0.05$ compared to control.

The above results suggest that CSQ2 knockdown may impair Ca^{2+} storage in C_2C_{12} myotubes thereby leading to reduced Ca^{2+} release. This possibility was further explored using pharmacological agents that empty Ca^{2+} stores in these myotubes but not through RyRs. In initial studies, we found that, in the absence of extracellular Ca^{2+}, neither adding UTP (an activator of IP_3Rs, 400 μM) following caffeine nor adding caffeine following UTP elicited an additional increase in Fluo-4 fluorescence in control and CSQ1/CSQ2 knockdown myotubes (not shown), thus suggesting that the two intracellular Ca^{2+} channel families release stored Ca^{2+} from the same stores. The effects of CSQ knockdown on IP_3R-mediated Ca^{2+} release were then determined by adding 400 μM UTP to the four groups of C_2C_{12} myotubes in Ca^{2+}-free KRH bath solutions. UTP elicited significantly less Ca^{2+} release in rAVV-CSQ2 and rAVV-CSQ1/rAVV-CSQ2-infected C_2C_{12} myotubes as compared with rAVV-control and rAVV-CSQ1-infected myotubes (Table 3). Consistently, in the absence of extracellular Ca^{2+}, thapsigargin (which empties intracellular Ca^{2+} stores by inhibiting SERCA1) also elicited significantly less Ca^{2+} responses in rAVV-CSQ2 and in rAVV-CSQ1/rAVV-CSQ2-infected C_2C_{12} myotubes (Table 3). We conclude that knocking down CSQ2 reduces SR Ca^{2+} stores and Ca^{2+} release in 8-day-old C_2C_{12} myotubes.

Immunoblot Analyses of C_2C_{12} Myotube Ca^{2+} Channels and Transporters—Because our Ca^{2+} transient measurements unexpectedly showed that CSQ2, but not CSQ1, is important for effective stored Ca^{2+} release in C_2C_{12} myotubes, we then undertook comprehensive immunoblot analyses to determine how knocking down each of the two CSQ isoforms may alter the expression of Ca^{2+} channels and transporters that may affect stored Ca^{2+} release in C_2C_{12} myotubes. The immunoblot analyses showed that the combined RyR (RyR1 and RyR3) protein levels in CSQ2 and CSQ1/CSQ2 knockdown myotubes were decreased by 27 ± 5 and 30 ± 6% ($n = 3$, $p < 0.05$), respectively, as compared with control and CSQ1 knockdown myotubes (Fig. 2*E*) ($n = 3$). SERCA1 protein levels were reduced by 50 ± 8 and 57 ± 7% ($n = 3$, $p < 0.05$) in CSQ2 and CSQ1/CSQ2 knockdown myotubes, respectively (Fig. 2*E*), as compared with the other two groups. In contrast, no differences in RyR3, IP_3R 1–3, triadin, junctin, or the α1 subunit of L-type Ca^{2+} channel (α1-DHPR) protein expression were detected among the four groups of C_2C_{12} myotubes, as compared with the actin control.

The decreased protein expression of RyRs was also supported by a ligand binding assay, using the RyR-specific probe ryanodine. B_{max} values of [^{3}H]ryanodine binding were similar in CSQ1 knockdown and control myotubes (Table 4). CSQ2 and CSQ1/CSQ2 knockdown myotubes had reduced B_{max} values of [^{3}H]ryanodine binding (0.21 and 0.24 pmol/mg of protein, respectively, *versus* control value of 0.35 pmol/mg of protein), which suggests a lower RyR protein expression as compared with control myotubes (Table 4). Most of this decrease could be accounted for by RyR1 (Fig. 2*E*).

Knocking Down CSQ2 but Not CSQ1 Reduces Ca^{2+} Uptake by the SR—To determine whether the decrease in SERCA1 protein expression among CSQ2 knockdown myotubes may also explain their reduced stored Ca^{2+} release, SERCA1 activities in the four different groups of myotubes were determined by measuring the $^{45}Ca^{2+}$ uptake rates of isolated membrane preparations. In agreement with the reduced SERCA1 protein levels (Fig. 2*E*), $^{45}Ca^{2+}$ uptake rates of membranes isolated from CSQ2 and CSQ1/CSQ2 knockdown myotubes were significantly lower than those of controls and CSQ1 knockdown myotubes (7.8 and 8.4 *versus* 18.6 and 16.5 nmol/mg protein/min, respectively) (Table 4). Thus, CSQ2 knockdown may impair Ca^{2+} storage in C_2C_{12} myotubes. In combination with the data presented in Table 3, we conclude that knocking down CSQ2 reduces cytosolic Ca^{2+} sequestration and thereby may reduce Ca^{2+} stores and Ca^{2+} release in 8-day-old C_2C_{12} myotubes.

DISCUSSION

In fast-twitch skeletal muscle CSQ2 is highly expressed during the fetal stage but then is gradually replaced by CSQ1 (20, 46). In slow-twitch skeletal muscle the two CSQ isoforms are co-expressed in adult animals (22, 47). X-ray crystallographic analyses have indicated that the two CSQ isoforms have almost identical crystal structures and form polymers as the Ca^{2+} concentration in the SR increases. Whether the two CSQ isoforms perform redundant or specific functions in skeletal muscle remains poorly understood. Herein we described an efficacious and specific knockdown method that allowed us to differentiate the roles of the two CSQ isoforms in stored Ca^{2+} release of skeletal muscle cell line C_2C_{12} myotubes. CSQ proteins in skeletal muscle have a half-life of ~5 days (49) and thus are difficult to knockdown once expressed. Accordingly, we infected C_2C_{12} myoblasts with rAVV vectors containing CSQ siRNA sequences before these cells mature into CSQ-expressing myotubes. Both CSQ proteins were absent or barely detectable in 1-day-old (control) myotubes but became easily detectable 3 (CSQ2) to 6 (CSQ1) days after forming myotubes. A similar time course of CSQ mRNA expression in maturing C_2C_{12} myotubes had been reported previously (46). Therefore, the high efficacies of CSQ knockdowns in our study are probably in part due to the timing of CSQ siRNAs delivery. Moreover, we found that knocking down one CSQ isoform did not decrease the expression nor caused a compensatory overexpression of the other isoform in C_2C_{12} myotubes. The high efficacies and specificities of our knockdown approach thus enable us to attribute the observed functional changes of C_2C_{12} myotubes to a specific CSQ knockdown rather than to the nonspecific effects of rAAV infection.

C_2C_{12} myotubes with knockdown of both CSQ isoforms provided membranes that contained RyR1 and RyR3 and associated proteins (triadin and junction) but depleted of CSQ proteins. Accordingly, we measured the single channel activities of skeletal RyRs in these membranes before and after adding individual CSQ isoform exogenously as previous reports have suggested that in striated muscles CSQ forms a tight multiprotein complex with RyRs and modulates the Ca^{2+} release of RyRs indirectly via a linkage protein, triadin. We found that both CSQ isolated from rabbit skeletal muscle (which is largely CSQ1) and CSQ isolated from canine heart (CSQ2) individually enhanced RyR channel opening probabilities in the presence of high lumenal Ca^{2+} concentrations (1 mM), although the effect of CSQ2 was much smaller but nevertheless statistically significant (adding 2 μM CSQ2 increased P_o from 0.24 ± 0.05 to 0.29 ± 0.06, $p < 0.05$). In contrast, adding rabbit skeletal muscle CSQ had no effect on the channel activities of RyRs in membranes isolated from control C_2C_{12} myotubes under identical conditions. These data indeed support the notion that CSQ proteins form a tight

protein complex with skeletal RyRs that rendered these channels from control C_2C_{12} myotubes insensitive to exogenously added CSQ. Moreover, because exogenously added CSQ proteins had no channel modulating effect on purified skeletal muscle RyRs, our data also support the notion that CSQ proteins (especially CSQ1) may indirectly facilitate stored Ca^{2+} release by skeletal RyRs via linkage protein(s) (50, 51).

It is therefore unexpected that we consistently found that knocking down CSQ2, but not CSQ1, reduced stored Ca^{2+} release in C_2C_{12} myotubes especially that in 8-day-old C_2C_{12} myotubes CSQ2 accounts for only one-third of CSQ proteins by densitometry analysis. We observed the reduction of stored Ca^{2+} release in CSQ2 knockdown (and double CSQ knockdown) myotubes caused by all three RyR activators that act on different skeletal RyR sites. It is therefore unlikely that knocking down CSQ2 reduces skeletal RyR Ca^{2+} release via specific allosteric mechanisms, such as disrupting the functional linkage between the DHPR and RyR1 required for activation of RyR1 by depolarization (KCl) (52). Moreover, we observed a similar reduction in stored Ca^{2+} release in CSQ2 knockdown myotubes induced by an activator of IP_3Rs (400 μM UTP) (48) and by thapsigargin (1 μM), an inhibitor of SERCA (Table 3). Taken together, these data suggest that knocking down CSQ2 leads to a reduction in Ca^{2+} storage in C_2C_{12} myotubes thereby leading to a general reduction in stored Ca^{2+} release.

In agreement with Beard *et al.* (18), lumenal CSQ did not affect single, purified single RyR1 channel activities or channels from control myotubes, presumably because CSQ remained with control channels. However, we found that RyR channels from CSQ1/CSQ2 knockdown C_2C_{12} myotubes were activated by purified skeletal and cardiac muscle CSQ, whereas CSQ inhibited the CSQ-depleted RyR1 channel complex (11). The reason for this discrepancy is not clear but could arise from different preparations and experimental conditions used by Beard *et al.* (11) and us. One difference was that skeletal SR vesicles predominantly contain RyR1 (18), whereas C_2C_{12} myotubes express RyR1 and RyR3. However, we observed an inactivation of CSQ-depleted channel activities by the purified CSQs in only 1 (of 18) single channel recording, which suggests that both RyR1 and RyR3 were likely activated by CSQ. There are also certain limitations in using high salt concentrations or knocking down CSQs. The use of high salt and Ca^{2+} concentrations might have resulted in an irreversible rearrangement of the RyR1 protein complex, an incomplete removal of CSQ, or loss of other proteins. CSQ knockdown may affect another yet to be identified protein involved in RyR and CSQ complex interaction, which could have resulted in the lower channel activities (P_o values) recorded with control membranes compared with membranes devoid of CSQ1/CSQ2. Whether CSQ modulates RyR activity directly or via triadin and junctin or an unidentified associated protein remains an open question.

Our immunoblot analyses suggest that the reduction in Ca^{2+} store size in CSQ2 knockdown myotubes is at least in part due to a reduction of expression of SERCA1 protein. The expression of SERCA1 protein in CSQ2 and CSQ1/CSQ2 knockdown myotubes is ~50% of the control and the CSQ1 knockdown myotubes. This reduction in SERCA1 had a large functional significance, as the Ca^{2+} uptake rate by SR vesicles prepared from CSQ2 or CSQ1/CSQ2 knockdown myotubes was also decreased by ~50%. At this point, the reduction of SERCA1 protein expression in CSQ2 knockdown myotubes remains unexplained. As the expression of other SR proteins, such as junctin, triadin, and RyR3, is mostly preserved in CSQ2 knockdown myotubes, it is unlikely that knocking down CSQ2 caused a general retardation of C_2C_{12} myotube maturation. However, it is notable that RyR1 expression appears to be decreased in CSQ2 knockdown myotubes. The reduction of RyR1 protein in CSQ2 knockdown myotubes is supported by a significant decrease in [^{3}H]ryanodine binding of membranes isolated from CSQ2 knockdown myotubes. The etiology and the functional significance of this reduction in RyR1 protein expression are currently unknown. To determine whether knocking down CSQ2 reduces SERCA1 and RyR1 protein expression in C_2C_{12} myotubes at the mRNA level, we are currently undertaking comprehensive quantitative real-time reverse transcriptase-PCR analyses of the four experimental C_2C_{12} myotube groups.

Effectively knocking down CSQ1 and CSQ2 in our study leads to a few unexpected findings that challenge the traditional views of the roles of these proteins in skeletal muscle. In particular, knocking down CSQ1 had no noticeable effects on Ca^{2+} storage and stored Ca^{2+} release in C_2C_{12} myotubes despite extensive prior data suggesting the roles of CSQ1 in buffering Ca^{2+} in the SR and in modulating RyR channel activities (4–9). In this study we have ruled out that CSQ1 knockdown leads to a compensatory overexpression of the other CSQ isoform thereby masking the functional roles of CSQ1 in stored Ca^{2+} release in C_2C_{12} myotubes. The next step in defining the functional roles of CSQ1 and CSQ2 would be the deletion of the two CSQ2 genes in skeletal muscle of mice and then study these animals for functional alterations in skeletal muscle. As a deficiency in CSQ2 protein may lead to fatal cardiac arrhythmia in young children (27), selective knock-out of CSQ2 in the skeletal muscle of mice may be necessary.

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